

## Hybrid proteins of active-site serine $\beta$ -lactamase

[0001] The present invention relates to hybrid proteins of active-site serine  $\beta$ -lactamase.

[0002] In the state of art a plurality of scientific publications have described the construction of fusion proteins. In the majority of these cases, such fusion proteins were realised as fusions of polypeptides to the N-terminus or C-terminus of a carrier protein. Martineau et al. (Martineau, P., J. G. Guillet, et al. (1992). "Expression of heterologous peptides at two permissive sites of the MalE protein: antigenicity and immunogenicity of foreign B-cell and T-cell epitopes." Gene 118(1): 151; Martineau, P., C. Leclerc, et al. (1996). "Modulating the immunological properties of a linear B-cell epitope by insertion into permissive sites of the MalE protein." Mol Immunol 33(17-18): 1345-58.) have realized the insertion of a protein into permissive sites of the protein MalE and they have studied the immune response against the inserted protein.

[0003] Several authors describe the immunisation against the enterotoxins STa (heat stable enterotoxin of E. coli) via constructing N-terminal or C-terminal fusion proteins, wherein different carrier proteins are involved, in order to obtain an immune response against the STa peptide, which as such is not immunogenic. The construction of hybrid proteins by inserting the STa peptide into a permissive site of a carrier protein has not yet been described in the prior art. The  $\beta$ -lactamase TEM-1 also not has been used as carrier protein for the construction of hybrid or fusion proteins with STa.

[0004] The process for synthesising bifunctional proteins got underway through binding one protein to another by chemical means. Against the background of this approach, the two proteins of interest, which have different properties, are synthesised independently and then treated with a chemical so as to achieve the covalent bonding of specific chemical groups available in the proteins. The technique has helped to achieve progress in developing diagnosis tests and recombinant vaccines. However, it has several drawbacks that have prompted the scientific community to develop other options. One of the disadvantages of using chemical agents is the aspecific coupling of two target proteins, which results in a lack of

uniformity in the way the two proteins are bound associated and oriented. This in turn may inactivate one of the proteins. As a result of the binding system, protein complexes are formed where the stoichiometry and composition is heterogeneous. For example, a protein X may be associated with one, two, three or more Y proteins. A binding between identical molecules (dimer-multimer) is difficult to avoid, thereby reducing the quantity of bifunctional proteins obtained. The resulting assay challenges can be intuitively understood, along with the calibration stage required to assess the sensitivity of the product with each further coupling.

[0005] Another challenge with the aspecific binding of the bridging agents is that these techniques call for large quantities of proteins in return for a reduced yield potential, thereby pushing up the costs of the finished product. A de novo synthesis of bifunctional proteins in prokaryotics or eukaryotics systems offers an alternative way of meeting these challenges. This method involves molecular biology techniques providing an opportunity to modify the structure of the coding gene for the proteins in question. However, this technology calls for a detailed knowledge of the biochemical and structural properties of the polypeptides synthesised on the basis of their manipulated sequence of nucleotides.

[0006] An initial de novo synthesis approach was adopted on the basis of fusion proteins. This involves genetically fusing coding DNA sequences for two proteins of interest to one or the other ends of the said genes. This fusion operation may apply to whole proteins, fragments of proteins or random peptides. The two proteins (or protein fragments) are then expressed in tandem by the producer organism. This technique solves the problems of difficult, insoluble, misfolding proteins. It also addresses issues related to chemical coupling (see above), even though it is not the technique by which other ones are judged in this sphere. Presenting a peptide to the ends of another protein means this protein is exposed to excessive proteolysis when the fusion protein is being produced and purified. What is more, the degrees of freedom of the fusion peptides are such that they seriously destabilise the structure of the entire fusion. The result is a total loss of biological activity.

[0007] Therefore it was object of the present invention to provide functional proteins wherein the respective carrier protein retains its activity and also the added

heterologous sequence still processes its function (for example as epitope, enzyme etc.), wherein furthermore the added heterologous sequence is somehow exposed on the surface of the carrier protein which is providing the possibility that the heterologous sequence may interact with other molecules. Furthermore, it was the object of the present invention to provide the functional proteins wherein the additional heterologous sequence maintains its free dimensional structure. It was a further object that the heterologous sequence is made less susceptible to proteolysis.

[0008] The object of the present invention is solved by a recombinant nucleotide sequence which codes upon expression for at least a part of a bifunctional hybrid active-site serine  $\beta$ -lactamase protein, wherein the  $\beta$ -lactamase protein is bearing at least one heterologous sequence, wherein the hybrid protein is having two functions, the first function is associated with the  $\beta$ -lactamase portion and the second function is associated with the heterologous sequence having a biological function which is different from the first function.

[0009] In a preferred embodiment the  $\beta$ -lactamase protein is having conserved amino acid elements 1, 2 and 3, wherein element 1 is having the amino acid sequence SXXK, element 2 is having the amino acid sequence SDN in class A proteins, YXN in class C proteins, SX[V or T or N] in class D proteins, wherein the elements of classes A, C and D correspond to each other, and element 3 is having the amino acid sequence K[T or S]G, wherein the  $\beta$ -lactamase protein is bearing at least one heterologous sequence between element 2 and element 3. Element 1 is the conserved sequence containing the serine of the active site and always located at the N-terminus of the  $\alpha$ 2 helix. Element 2 is the SDN loop of class A, corresponding to YXN and SX[V or T or N] in class C and D, respectively. This loop is located between helices  $\alpha$ 4 and  $\alpha$  5 in class A  $\beta$ -lactamases. In class A, the serine seems to be involved in maintaining the functional positioning of the two domains (the all  $\alpha$  and the  $\alpha$ /beta domains). The tyrosine at position 150 in class C  $\beta$ -lactamase could act as a general base in the catalytic phenomenon. Element 3 is located on the  $\beta$ -strand domain of the  $\beta$ -lactamase facing the second element.

[0010] Further preferred, the  $\beta$ -lactamase protein is bearing at least one heterologous sequence in a region located between two neighboring  $\alpha$  helices of the  $\beta$ -lactamase sequence, wherein the region is forming a juncture between the

alpha helices of active-site serine  $\beta$ -lactamases, wherein said alpha helices correspond to the last two alpha helices before the alpha/beta domain. The active-site serine  $\beta$ -lactamases of classes A, C and D have a two domain structure: one domain is the alpha domain containing alpha helices only; the other domain is the alpha/beta domain containing alpha helices and beta sheets. The loop between the last two alpha helices of the first domain (the all alpha helices domain) is the preferred insertion site of the heterologous sequence according to the present invention. In TEM-1  $\beta$ -lactamase, which is a class A  $\beta$ -lactamase, these last two alpha helices of the all alpha domain are alpha helix 8 and alpha helix 9, which are before the alpha/beta domain. Due to the high similarity between class A, C and D  $\beta$ -lactamases on a three-dimensional level, these helices and their loop in between (as insertion site) can be identified in any  $\beta$ -lactamase of any of the three classes A, C and D.

[0011] Furthermore, it is preferred the  $\beta$ -lactamase protein is bearing at least one heterologous sequence in a region located between two neighboring alpha helices of the  $\beta$ -lactamase sequence, wherein the region is selected from:

- a) the region forming a juncture between alpha helix 8 and alpha helix 9 of TEM-1  $\beta$ -lactamase;
- b) the region forming a juncture between the alpha helices which are homologous to alpha helix 8 and alpha helix 9 of TEM-1  $\beta$ -lactamase.

[0012] In another preferred embodiment the  $\beta$ -lactamase moiety is selected from the group:

- a) class A  $\beta$ -lactamase,
- b) class C  $\beta$ -lactamase,
- c) class D  $\beta$ -lactamase,
- d) a recombinant sequence of one or more of a) to c).

[0013] In one alternative embodiment the  $\beta$ -lactamase moiety is derived from class A  $\beta$ -lactamase, wherein  $\beta$ -lactamase class A protein is bearing the heterologous sequence in the region forming a juncture between alpha helix 8 and

alpha helix 9. More preferred the region forming a juncture between alpha helix 8 and alpha helix 9 is selected from the group:

- a) the amino acid sequence Thr195 to Leu199 of the TEM-1  $\beta$ -lactamase;
- b) the amino acid sequence corresponding to the amino acid sequence Thr195 to Leu199 in TEM-1  $\beta$ -lactamase.

[0014] The amino acid sequence corresponding to the amino acid sequence Thr195 to Leu199 in TEM-1  $\beta$ -lactamase is located between the last two alpha helices of the all alpha domain. In class A  $\beta$ -lactamase these helices are helix 8 and helix 9. The alpha helices 8 and 9 are defined as sequences ARALATSLQAFA and SEKRELLIDWMK in BlaP and are defined as PAAMATTLRKLL and LASRQQLIDWME in TEM-1  $\beta$ -lactamases, respectively, and those alpha helices which correspond to those in  $\beta$ -lactamases of the same class or of classes C and D.

[0015] In another alternative embodiment the  $\beta$ -lactamase moiety is derived from class C  $\beta$ -lactamase, wherein  $\beta$ -lactamase class C protein is bearing the heterologous sequence in the region forming a juncture between alpha helices, which correspond to alpha helix 8 and alpha helix 9 in TEM-1  $\beta$ -lactamase.

[0016] In a further alternative embodiment the region forming a juncture is selected from the group:

- a) the amino acid sequence K239 to E245 of the AmpC  $\beta$ -lactamase;
- b) the amino acid sequence corresponding to the amino acid sequence K239 to E245 of the AmpC  $\beta$ -lactamase.

[0017] The amino acid sequence corresponding to the amino acid sequence K239 to E245 of the AmpC  $\beta$ -lactamase is located between the last two alpha helices of the all alpha domain. In class C  $\beta$ -lactamase these helices, which correspond to helix 8 and helix 9 of class A  $\beta$ -lactamases, are defined as sequences IEDMARWVQSNL and KTLQQGIQLA, respectively, in AmpC, and defined by alpha helices which correspond to those in  $\beta$ -lactamases of the same class or of classes A and D.

[0018] In a further alternative embodiment the  $\beta$ -lactamase moiety is derived from class D  $\beta$ -lactamase, wherein  $\beta$ -lactamase class D protein is bearing the heterologous sequence in the region forming a juncture between alpha helices, which correspond to alpha helix 8 and alpha helix 9 in TEM-1  $\beta$ -lactamase. In a preferred embodiment the region forming a juncture is selected from the group:

- a) the amino acid sequence N510 to Q516 of the BlaR-CTD  $\beta$ -lactamase;
- b) the amino acid sequence corresponding to the amino acid sequence N510 to Q516 of the BlaR-CTD  $\beta$ -lactamase.

[0019] The amino acid sequence corresponding to the amino acid sequence N510 to Q516 of the BlaR-CTD  $\beta$ -lactamase. In class D  $\beta$ -lactamase these helices, which correspond to helix 8 and helix 9 of class A  $\beta$ -lactamases, are defined as sequences SPLEQVNIKKFYD and KQSNIETVKDSI, respectively, in BlaR-CTD, and defined by alpha helices which correspond to those in  $\beta$ -lactamases of the same class or of classes A and C.

[0020] The following table is showing the positions of the two helices helix 8 and helix 9 in different  $\beta$ -lactamases of class A, C and D enzymes.

**Table: 1** Comparison of corresponding helices 8 and 9 in  $\beta$ -lactamases. The numbering scheme for amino acids is according to ABL (see text).

|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                 | Helix 8 (grey highlight) |         | Helix 9 (underlined only) |         | Example for an insertion site |
|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------|---------|---------------------------|---------|-------------------------------|
|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                 | start                    | end     | start                     | end     |                               |
| BlaP                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            | Ala 183                  | Ala 194 | Ser 201                   | Lys 212 | Asp 197                       |
| TEM-1                                                                                                                                                                                                                                                                                                                                                                                                                                                                                           | Pro 183                  | Leu 194 | Leu 201                   | Glu 212 | Glu 197                       |
| AmpC                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            | Ile 227                  | Leu 238 | Lys 246                   | Ala 255 | Leu 241                       |
| BlaR-CTD                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        | Ser 496                  | Asp 509 | Lys 515                   | Ile 526 | Phe 514                       |
| <b>BlaP (bold: signal peptide)</b><br>mklwfstlkl kkvaavllfs cvalagcgsn hsnashsaek dektemkddf akleeqfdak<br>lgifaltdgt nrtvtlyrpd rfafastika ltvgvllqqk siedlnqrit ytrddlvnyn<br>pitekhvdtg mtlkeladas lrysdntagn lilkgigppe slkkelrkig devtnperfe<br>pelnevnpgd tqdtstaral <u>atslgafale</u> dklpsekrel lidwmkrntt gdaliragvp<br>egwevadktg agsygtrndi aiiwppkgdp vvlavlssrd kkdakyddkl iaeatkvvvk<br>alnmesk                                                                                   |                          |         |                           |         |                               |
| <b>TEM-1 (bold: signal peptide)</b><br>msiqhfrval ipffaafclp vfahpetlvk vkdaedqlga rvgyieldln sgkilesfrp<br>eerfpmmstf kvllcgavls rvdaggeqlg rrihysqndl veyspvtekh ltdgmtvrel<br>csaaitsmsn taanllltti ggpkeltafl hnmgdhvtl drwepelnea ipnderdttm<br>paamattlrk <u>lltgelltla</u> <u>srqqlidwme</u> adkvagpll salpagwfia dksgagergs<br>rgiiaalgpd gkpsrivviy ttgsqatmde rnrqiaeiga slikhw                                                                                                       |                          |         |                           |         |                               |
| <b>AmpC (bold: signal peptide)</b><br>mfkttlcall itascstfaa pqqindivhr titplieqqk ipgmavaviy qgkpyyftwg<br>yadiakkqp tqqtlfelgs vsktftgvlq gdaiargeik lsdpttkywp eltakqwnqi<br>tllhlatyta gglplqvpde vkssdllrf yqnwqpawap gtqrlyanss iglfgalavk<br>psglsfegam qtrvfqplkl nhtwinvppa eeknyawgyr egkavhvspg aldaeygvk<br>stiedmarwv <u>genlkpldin</u> <u>ektlqqgiql</u> aqsrywtgd myqglgweml dwpvnpsii<br>ngsdnkiala arpvkaitpp tpavraswvh ktgatggfgs yvafipekel givmlankny<br>pnparvdaaw qilnalq |                          |         |                           |         |                               |
| <b>BlaR-CTD</b><br>346 MQKET<br>351 RFLPGTNVEY EDYSTFFDKF SASGGFVLFN SNRKKYTIYN RKESTSRFAP<br>401 ASTYKVFSAL LALESGIITK NDSHMTWDGT QYPYKEWNQD QDLFSAMSSS<br>451 TTWYFQKLD R QIGEDHLRHY LKSIHYGNED FSVPADYWLD GSLQISPLEQ<br>501 <u>VNTLKKFYDN</u> EFDFKQSNIE TVKDSIRLEE SNGRVLGKT GTSVINGELH<br>551 AGWFIGYVET ADNTFFFAVH IQGEKRAAGS SAAEIALSIL DKKGIYPSVS<br>601 R                                                                                                                              |                          |         |                           |         |                               |

[0021] The numbering scheme of the amino acids sequences in table 1 is according to Amber, R. P., A. F. Coulson, J. F. Frère, J. M. Ghuysen, M. Forsman, B. Joris, R. Levesque, G. Tiraby, and S. G. Waley. 1991. A standard numbering scheme for the class A  $\beta$ -lactamases. Biochem. J. 276: 269-270. The BlaR protein is organized as a two-domain protein, including an N-terminal domain [BlaR-NTD, from residues 1 to 345] anchored into the membrane and an extracellular C-terminal domain [BlaR-CTD, from residues 346 to 601]. This latter, belongs to the serine penicillin-recognizing protein family and display the same 3-dimensional structure as class A  $\beta$ -lactamase. This is the reason why the sequence presented in table 1 begins at position 346.

[0022] The object of the present invention is also solved by a recombinant nucleotide acid sequence which codes upon expression for at least a part of a bifunctional hybrid  $\beta$ -lactamase class A protein, wherein the  $\beta$ -lactamase class A protein is bearing at least one heterologous sequence in a region located between two neighbouring alpha helices of the  $\beta$ -lactamase sequence, wherein the region is selected from:

- a) the region forming a juncture between alpha helix 8 and alpha helix 9 of the TEM-1  $\beta$ -lactamase;
- b) the region forming a juncture between the alpha helices of homologous  $\beta$ -lactamases class A, said alpha helices corresponding to the alpha helix 8 and alpha helix 9 of the TEM-1  $\beta$ -lactamase.

[0023] In a preferred embodiment the hybrid  $\beta$ -lactamase is possessing an activity selected from

- a) hydrolysing  $\beta$ -lactams;
- b) binding covalently and in a stable manner to derivatives of  $\beta$ -lactams and inhibitors of  $\beta$ -lactamases.

[0024] In a further preferred embodiment the hybrid protein is having two functions, the first function is associated with the  $\beta$ -lactamase portion and is selected from

- a) hydrolyzing  $\beta$ -lactams ( $\beta$ -lactamase activity);



b) binding covalently and in a stable manner to substances selected from the group  $\beta$ -lactams, derivatives of  $\beta$ -lactams, inhibitors of  $\beta$ -lactams; wherein the second function is associated with the heterologous sequence having a biological function which is different from the first function.

[0025] According to the present invention the  $\beta$ -lactamase portion retains its activity, even after the homologous sequences is inserted. Although the degree of the activity might vary, the  $\beta$ -lactamase portion will have the same kind of activity as before the insertion. For example, if the  $\beta$ -lactamase was able to catalyse the cleavage of  $\beta$ -lactams, this activity will be maintained even after insertion. If a mutant  $\beta$ -lactamase is used which binds covalently to  $\beta$ -lactams and/or derivatives thereof, but does not catalyse the cleavage completely, then also this kind of mutant activity will be maintained after insertion of the heterologous sequence. The latter activity is useful to immobilize  $\beta$ -lactamase and hybrid  $\beta$ -lactamase, respectively, on carriers containing bound  $\beta$ -lactam substances.

[0026] Furthermore it is preferred that the hybrid  $\beta$ -lactamase retains its activity of hydrolysing  $\beta$ -lactams at least partially.

[0027] **Detailed Description of the Invention**

[0028] The technological advance offered by the present invention involves internalising the proteins or polypeptide fragments within the native structure of a carrier protein, an active-site serine  $\beta$ -lactamase. This new approach provides a means of replacing the internalised fragments in a 3-dimensional context close to the native situation. The constraints of the carrier protein imposed compel the internalised peptides to adopt a proper structure. This guarantees continuing biological activities in many cases. The outcome is the creation of a bifunctional hybrid protein. The hybrid  $\beta$ -lactamase according to the invention represents a single polypeptide. The use of this hybrid  $\beta$ -lactamase preferably is to study the interaction of the internalised homologous sequence with other separate molecules (for example antibodies) or to use this interaction of the internalised homologous sequence with other separate molecules in assays, either as binding interaction to be measured or as part of the test.

[0029] As mentioned above the hybrid  $\beta$ -lactamase according to the present invention is a single polypeptide. According to the present invention this single polypeptide exerts two biological functions: the first function is derived from the  $\beta$ -lactamase carrier as used. The second function is associated with the heterologous sequence. As a consequence the hybrid  $\beta$ -lactamase according to the invention combines the function of its basic constituents in one single peptide: the one function is that of the  $\beta$ -lactamase carrier and the other function is that of the heterologous sequence.

[0030]  $\beta$ -lactamase enzymes are divided into four classes. Classes A, C and D gather together the active serine enzyme. The fold of these three classes share a very close structure which is characterized by two domains. An  $\alpha$  helix domain and a domain containing  $\alpha$  helices and  $\beta$  sheets (see figure 1). In contrast class B  $\beta$ -lactamase are metallo- $\beta$ -lactamases which need zinc ions to catalyse the  $\beta$ -lactam hydrolysis. The 3-dimensional structure of these class B enzymes has no common structure compared to the three other  $\beta$ -lactamases of class A, C and D.

[0031] The inventors constructed several hybrid proteins by inserting restriction sites in the DNA sequences of the  $\beta$ -lactamase in TEM-1 (*E. coli*), BlaL (*Streptomyces cacaoi*) and BlaP (*Bacillus licheniformis*) (class A). Once hybrid proteins on the basis of TEM-1, BlaL, BlaP have been successfully constructed further experiments with enzymes of other classes have been performed. Based on the structure of AmpC and by random insertions in BlaR-CTD, the restriction sites were inserted in a region common to the class A  $\beta$ -lactamase BlaP so as to be able to retain  $\beta$ -lactamase activity after insertion of large exogenous sequences. By that hybrid proteins based on AmpC from *E. coli* K12 (class C) and in BlaR-CTD from *Bacillus licheniformis* (class D), respectively, have also been constructed.

[0032] The inventors internalised exogenous nucleotide sequences in these genes. The hybrid genes produced during these operations provide a means of producing various bifunctional proteins.

[0033] The catalytic efficiency and the plasticity of the class A, C and D  $\beta$ -lactamases means they are effective candidates for constructing bifunctional

proteins as a result of inserting sequences of exogenous peptides within the enzyme structure.

[0034] The TEM-1  $\beta$ -lactamase is an active class A serine enzyme involved in the bacterial resistance to  $\beta$ -lactam based antibiotics, such as penicillin and cephalosporins. The mature form of the TEM-1  $\beta$ -lactamase is a monomeric protein with 263 amino acids. Its 3D structure is hallmarked by two areas one of which is helice-rich and the other comprises  $\alpha$  helices and  $\beta$  sheets (figure 1).

[0035] The class A  $\beta$ -lactamases interact with the  $\beta$ -lactam based antibiotics to form an intermediate called acyl-enzyme where the antibiotic is covalently linked to the serine of the enzyme's active site (figure 2). The  $\beta$ -lactamases efficiently catalyse the deacylation stage (k3). This regenerates the active enzyme and releases a biologically inactive substance, where the amide linkage of the  $\beta$ -lactam of the antibiotic's nucleus is hydrolysed. In the case of the TEM-1  $\beta$ -lactamase mutagenesis, experiments have shown that the deacylation reaction could be inhibited to produce a stable acyl-enzyme complex by replacing the glutamate 166 residues in asparagine (6) (figure 2). This feature permits the immobilisation of TEM-1  $\beta$ -lactamase hybrid protein on  $\beta$ -lactam coated matrix.

[0036] As a result of the specificity, catalytic efficiency and plasticity of the TEM-1  $\beta$ -lactamase, this protein is a valuable enzyme for developing new processes for detecting, assaying and orienting peptides and proteins used for therapeutic purposes. Towards this end, chromogenic and fluorescents substrates, suicide inhibitors and prodrug cytotoxic agents have been developed.

[0037] Transposition and phage display experiments have shown the possibility of introducing or degenerating very short sequences of nucleotides (8 to 30 nucleotides) in the coding sequence for the TEM-1  $\beta$ -lactamase and using these mutated genes as a basis for synthesizing a constantly functional enzyme (3, 5, 14, 15). In the context of this technology (WO98/23731) phage libraries ( $10^{10}$  phages) bearing chimeras of the TEM-1  $\beta$ -lactamase, where small degenerated peptides have been inserted, are used to select the degenerated peptide that has an affinity for a given target. This is a cumbersome, painstaking and evolutionary method, as it is not

common for a peptide having a high affinity for the target to be selected. This characteristic involves creating several mutagenesis stages in respect of the peptide and the carrier protein so as to optimise the affinity of the hybrid protein. Hence this entails creating new banks and producing new screenings.

[0038] The idea of the present invention is not to create a chimera bank that theoretically covers all types of biodiversity but favours the insertion of large peptidic sequences present in proteins whose biochemical characteristics have already been clearly identified. Consequently, the present invention provides a means of internalising a peptidic sequence that has already been naturally optimised for a given property and averting (as is the case with the phage display technique) evolutionary mutagenesis reactions within the insert and carrier protein. With this system, the chimera banks may be restricted to a few thousand clones (or a few dozens) so the screening is quicker and more targeted.

[0039] Unlike the results obtained with the various TEM-1  $\beta$ -lactamase utilisation methods, wherein the insertion site leads to a change in the TEM-1 enzymatic properties, this present invention marks a new departure because the inventors succeeded in identifying and creating, in a loop diametrically opposed to the active enzyme site (figure 1, site B: region Thr195 to Leu199), a region that is particularly favourable for internalising large exogenous sequences of peptides with a length preferably 11 or more amino acids. In this application this loop Thr195 to Leu199 is also referred to as the region forming the juncture between alpha helix 8 and alpha helix 9 of the TEM-1  $\beta$ -lactamase and homologous enzymes which have a homologous three-dimensional structure, for example like BlaP and BlaL. The present invention applies to hybrid  $\beta$ -lactamase proteins wherein the carrier is selected from  $\beta$ -lactamases of classes A, C and D. Consequently, the present invention refers to hybrid  $\beta$ -lactamases wherein the carrier is an active-site serine  $\beta$ -lactamase. According to the present invention the heterologous sequence to be internalised is a sequence different from  $\beta$ -lactamases or parts thereof.

[0040] The results show that the polypeptides internalised in the context of the above identified loop are able to adopt a folding close to their native conformation, as their biological activity is retained. This specific feature offers new prospects for constructing and using new generations of hybrid bifunctional proteins where a

specific and effective enzymatic activity is associated with the biochemical properties of another protein or a fragment of a protein. This special feature is the second focal point of this invention, as it is of crucial importance for developing the diagnosis test or the chromatography affinity system, because the  $\beta$ -lactamase activity is used to quantify the protein-macromolecule interaction or to immobilise the hybrid protein in an oriented way.

[0041] As described in the patent application WO 98/23731 the TEM-1 enzyme was shown to be sensitive to the action of proteases during production in bacterial system, which reduces the output of hybrid proteins. By applying the technology according to the present invention also to  $\beta$ -lactamases that are more resistant to proteases (BlaP and BlaL) further advantageous constructs have been provided. These BlaP and BlaL  $\beta$ -lactamases are produced by Gram positive organisms for which suitable production tools exist. Furthermore, these bacteria are well-known in industry and enjoy GRAS (Generally Regarded As Safe) status. In the case of BlaP and BlaL it was possible to show that polypeptide insertion technique could be transferred to other  $\beta$ -lactamases of the same class and make a generally well-known improvement to the technology via the properties specific to these enzymes. The inventors also proved that the technique can be applied to  $\beta$ -lactamases of classes C and D.

[0042] In a further preferred embodiment the nucleotide sequence coding for the  $\beta$ -lactamase sequence is selected from:

- a) nucleotide sequence coding for the  $\beta$ -lactamase TEM-1 (SEQ ID NO: 1; complementary strand);
- b) nucleotide sequence coding for the  $\beta$ -lactamase BlaP (SEQ ID NO: 2);
- c) nucleotide sequence coding for the  $\beta$ -lactamase BlaL (SEQ ID NO: 3);
- d) nucleotide sequence coding for the  $\beta$ -lactamase AmpC (SEQ ID NO: 39);
- e) nucleotide sequence coding for the  $\beta$ -lactamase BlaR-CTD (SEQ ID NO: 41);
- f) nucleotide sequences which hybridise under stringent conditions to the nucleotide sequences of any one of a) to e) or fragments thereof. It should be noted that the nucleotide sequence SEQ IDNO: 1 given for the  $\beta$ -lactamase TEM-1 here is the complementary strand.

[0043] In a further preferred embodiment the heterologous sequence is partially or fully replacing the region between alpha helix 8 and alpha helix 9 or the region between alpha helix 9 and alpha helix 10. As described above the numbering of the helices refers to the TEM-1  $\beta$ -lactamase. The present invention also includes homologous  $\beta$ -lactamases of class A, so that the identification of helix 8 and helix 9 in those homologous enzymes has to be applied in a corresponding manner. In case of the TEM-1  $\beta$ -lactamase this region is defined by the amino acid residues Thr195 – Leu199. The present invention further relates to  $\beta$ -lactamases of classes C and D which correspond to class A  $\beta$ -lactamases in respect to the three-dimensional structure. Therefore, in all active-site serine  $\beta$ -lactamases there can be identified helices which correspond to helix 8 and helix 9 of TEM-1  $\beta$ -lactamase.

[0044] In a further preferred embodiment the heterologous sequence has a length of 11 or more amino acid residues, preferably in the range of 11 to 5000 amino acid residues, more preferably in the range of 11 to 3000 amino acid residues, more preferred in the range of 11 to 2000 amino acid residues, and further preferred in the range of 11 to 1000, even more preferred in the range of 11 to 300, and most preferred in the range of 18 to 200 amino acid residues. According to the present invention the insertion site within the  $\beta$ -lactamase class A, C or D for the heterologous sequence is determined in that way that any sequence of any length can be inserted essentially without disturbing the three-dimensional structure and the activity of the  $\beta$ -lactamase (also the so called “carrier protein”), as shown below.

[0045] In an alternative embodiment the heterologous sequence has a length of 18 or more amino acid residues, preferably in the range of 18 to 5000 amino acid residues, more preferably in the range of 18 to 3000 amino acid residues, more preferred in the range of 18 to 2000 amino acid residues, further preferred in the range of 18 to 1000, even more preferred in the range of 18 to 300, most preferred in the range of 18 to 200 amino acid residues.

[0046] In a further alternative embodiment the heterologous sequence has a length of 25 or more amino acid residues, preferably in the range of 25 to 5000 amino acid residues, more preferably in the range of 25 to 3000 amino acid residues, more preferred in the range of 25 to 2000 amino acid residues, further preferred in the

range of 25 to 1000, even more preferred in the range of 25 to 300, most preferred in the range of 25 to 200 amino acid residues.

[0047] In yet another alternative embodiment the heterologous sequence has a length of 50 or more amino acid residues, preferably in the range of 50 to 5000 amino acid residues, more preferably in the range of 50 to 3000 amino acid residues, more preferred in the range of 50 to 2000 amino acid residues, further preferred in the range of 50 to 1000, even more preferred in the range of 50 to 300, most preferred in the range of 50 to 200 amino acid residues.

[0048] In still another alternative embodiment the heterologous sequence has a length of 18 or more amino acid residues, preferably in the range of 100 to 5000 amino acid residues, more preferably in the range of 100 to 3000 amino acid residues, more preferred in the range of 100 to 2000 amino acid residues, further preferred in the range of 100 to 1000, even more preferred in the range of 100 to 300, most preferred in the range of 100 to 200 amino acid residues.

[0049] According to the present invention the hybrid  $\beta$ -lactamase is a bifunctional protein. Since the carrier protein, the  $\beta$ -lactamase moiety of the hybrid  $\beta$ -lactamase retains its activity and also the heterologous sequence originally also has a kind of function (for example as an epitope) the hybrid  $\beta$ -lactamase possesses two functions and therefore is a bifunctional protein.

[0050] The heterologous sequence is related to a function on the level of the peptide/polypeptide. According to the invention the function of the heterologous sequence does not refer to its mere physical presence. In the sense of the invention the heterologous (peptide/protein) sequence does not have only a mere structural function but goes beyond that. The term "function" or "biological function" of the heterologous sequence as used herein means that the inserted heterologous peptide or polypeptide is able to specifically interact with or recognize other substances or compounds, for example a substrate or for example a biological macromolecule, for example by way of an epitope – antibody interaction. The interaction or recognition exerted by the heterologous sequence preferably refers to a molecule different from the hybrid  $\beta$ -lactamase itself or parts thereof. Consequently, the function of the

heterologous sequence preferably involves specific interaction with other molecules (either low molecular compounds or macromolecules, for example biological macromolecules, for example peptides, proteins, nucleic acids). In particularly preferred embodiments the function of the heterologous sequence as such is selected from: being an epitope, being a specific binding partner for antibodies, being specially recognised and bound by antibodies, having a binding affinity to earth alkaline ions and metal ions, having enzymatic activity, being a toxin (for example STa heat-stable enterotoxin of *E. coli*), bearing a glycosylation site, bearing a glycosylated peptide, being a specific binding partner for any polypeptide or any ligand, having a binding affinity to dsDNA and ssDNA or RNA (having a binding affinity to nucleotide and polynucleotide).

[0051] Furthermore, it is particularly preferred that the heterologous sequence is selected from the group: STa (heat stable enterotoxin of *Escherichia coli*, SEQ ID NO: 21), protein A of *Staphylococcus aureus*, (SEQ ID NO: 23 and 25), protein G of *Streptococcus pyogenes*, (SEQ ID NO: 27 and 29), a linear antigenic determinant of the hemagglutinin of the Influenza virus (SEQ ID NO: 31), a fragment of human phospholipase – type II (hPLA<sub>2</sub>) (SEQ ID NO: 33), LPS binding amino acid sequence (SEQ ID NO: 35), and nucleotide sequences which hybridise under stringent conditions to said nucleotide sequences or fragments thereof.

[0052] Furthermore the present invention provides a recombinant polypeptide which is encoded by the recombinant nucleotide sequence as described before.

[0053] The present invention therefore provides a recombinant polypeptide comprising at least a part of a bifunctional hybrid active-site serine  $\beta$ -lactamase protein, wherein the  $\beta$ -lactamase protein is bearing at least one heterologous sequence, wherein the hybrid protein is having two functions, the first function is associated with the  $\beta$ -lactamase portion and the second function is associated with the heterologous sequence having a biological function which is different from the first function.

[0054] Preferably, the  $\beta$ -lactamase protein is having conserved amino acid elements 1, 2 and 3, wherein element 1 is having the amino acid sequence SXXK,



element 2 is having the amino acid sequence SDN in class A proteins, YXN in class C proteins, SX[V or T or N] in class D proteins, wherein the elements of classes A, C and D correspond to each other, and element 3 is having the amino acid sequence K[T or S]G, wherein the  $\beta$ -lactamase protein is bearing at least one heterologous sequence between element 2 and element 3.

[0055] In a preferred embodiment the  $\beta$ -lactamase protein is bearing at least one heterologous sequence in a region located between two neighboring alpha helices of the  $\beta$ -lactamase sequence, wherein the region is forming a juncture between the alpha helices of active-site serine  $\beta$ -lactamases, wherein said alpha helices correspond to the last two alpha helices before the alpha/beta domain.

[0056] In yet another embodiment the  $\beta$ -lactamase protein is bearing at least one heterologous sequence in a region located between two neighboring alpha helices of the  $\beta$ -lactamase sequence, wherein the region is selected from:

- a) the region forming a juncture between alpha helix 8 and alpha helix 9 of TEM-1  $\beta$ -lactamase;
- b) the region forming a juncture between the alpha helices which are homologous to alpha helix 8 and alpha helix 9 of TEM-1  $\beta$ -lactamase.

[0057] In a further embodiment the  $\beta$ -lactamase moiety is selected from the group:

- a) class A  $\beta$ -lactamase,
- b) class C  $\beta$ -lactamase,
- c) class D  $\beta$ -lactamase,
- d) a recombinant sequence of one or more of a) to c).

[0058] In one alternative the  $\beta$ -lactamase moiety is derived from class A  $\beta$ -lactamase, wherein  $\beta$ -lactamase class A protein is bearing the heterologous sequence in the region forming a juncture between alpha helix 8 and alpha helix 9.

[0059] Preferably the region forming a juncture between alpha helix 8 and alpha helix 9 is selected from the group:

- a) the amino acid sequence Thr195 to Leu199 of the TEM-1  $\beta$ -lactamase;

b) the amino acid sequence corresponding to the amino acid sequence Thr195 to Leu199 in TEM-1  $\beta$ -lactamase.

[0060] In another alternative the  $\beta$ -lactamase moiety is derived from class C  $\beta$ -lactamase, wherein  $\beta$ -lactamase class C protein is bearing the heterologous sequence in the region forming a juncture between alpha helices, which correspond to alpha helix 8 and alpha helix 9 in TEM-1  $\beta$ -lactamase.

[0061] Preferably, the region forming a juncture is selected from the group:

- a) the amino acid sequence K239 to E245 of the AmpC  $\beta$ -lactamase;
- b) the amino acid sequence corresponding to the amino acid sequence K239 to E245 of the AmpC  $\beta$ -lactamase.

[0062] In yet another embodiment the the  $\beta$ -lactamase moiety is derived from class D  $\beta$ -lactamase, wherein  $\beta$ -lactamase class D protein is bearing the heterologous sequence in the region forming a juncture between alpha helices, which correspond to alpha helix 8 and alpha helix 9 in TEM-1  $\beta$ -lactamase.

[0063] Preferably, the region forming a juncture is selected from the group:

- a) the amino acid sequence N510 to Q516 of the BlaR-CTD  $\beta$ -lactamase;
- b) the amino acid sequence corresponding to the amino acid sequence N510 to Q516 of the BlaR-CTD  $\beta$ -lactamase.

[0064] Furthermore the present invention provides a recombinant polypeptide which is encoded by the recombinant nucleotide sequence as described before. The present invention therefore provides a recombinant polypeptide comprising at least a part of a bifunctional hybrid  $\beta$ -lactamase class A protein, wherein that the  $\beta$ -lactamase class A protein is bearing at least one heterologous sequence in a region located between two neighboring alpha helices of the  $\beta$ -lactamase sequence, wherein the region is selected from:

- a) the region forming a juncture between alpha helix 8 and alpha helix 9 of the TEM-1  $\beta$ -lactamase;
- b) the region forming a juncture between the alpha helices, which correspond to the alpha helix 8 and alpha helix 9 of the TEM-1  $\beta$ -lactamase.

[0065] The further preferred embodiments of the recombinant polypeptides are outlined in respect to the description of the respective nucleotide sequence encoding the hybrid  $\beta$ -lactamase.

[0066] Furthermore, the present invention provides the use of the recombinant nucleotide sequence of the recombinant polypeptide for vaccination. As described above the enterotoxin of *Escherichia coli* (STa) as such is not immunogenic. However, by incorporating this peptide into a  $\beta$ -lactamase class A protein, namely in a region on the surface of this carrier protein, the possibility is given to raise antibodies against the heterologous sequence (STa), as shown below. Therefore, it is also preferred to use the recombinant nucleotide sequence or the recombinant polypeptide of the present invention for raising antibodies against the heterologous sequence. A further preferred embodiment is the use of the same for epitope mapping for a different protein or polypeptide. For epitope mapping smaller peptides having for example a length of 5 to 30 amino acid residues which are covering the polypeptide to be examined are used for being introduced as heterologous sequence in the region forming a juncture between alpha helix 8 and alpha helix 9 of the TEM-1  $\beta$ -lactamase or homologs thereof. As result a set of hybrid  $\beta$ -lactamase class A protein is constructed bearing (overlapping) sequences as heterologous sequence of the polypeptide to be examined. This set of hybrid  $\beta$ -lactamases differing within the homologous sequence is then used for studying the epitopes of the polypeptide in question can be studied.

[0067] The present invention also provides the use of the recombinant nucleotide sequence or the recombinant polypeptide of the present invention for affinity chromatography, particularly for the concentration and/or purification of antibodies directed against the heterologous sequence of the hybrid  $\beta$ -lactamase. By using a mutant of the  $\beta$ -lactamase class A protein (glutamate 166 asparagine) the  $\beta$ -lactamase can be immobilised on a matrix which is coated with the substrate ( $\beta$ -lactam). Since the hybrid  $\beta$ -lactamase is presenting the heterologous sequence on its surface it is possible to concentrate and/or purify antibodies which are directed against this heterologous sequence, which is immobilised on for example a column via the  $\beta$ -lactamase carrier protein, which is linked to its substrate on the matrix of the column.

[0068] In a similar way it is possible to detect molecules which are binding to the heterologous sequence qualitatively and/or quantitatively. It is particularly preferred that the molecules binding to the heterologous sequence are antibodies or antibody fragments, polypeptides, dsDNA, ssDNA, RNA or small ligands. The method which may apply for the qualitative and/or quantitative detection is well known as ELISA.

[0069] Furthermore the recombinant polypeptide preferably may be used in molecular diagnostics. For example, the protein A of *Staphylococcus aureus* or protein G of *Streptococcus pyogenes* or fragments thereof may be used as heterologous sequence incorporated into  $\beta$ -lactamase A protein. This heterologous sequence is exposed on the surface of the carrier protein ( $\beta$ -lactamase class A) and binds to the Fc region of antibodies. By using substrates of the  $\beta$ -lactamase which upon cleavage show a colour change, this system can be applied for the quantitative and/or qualitative detection of antigens to which the antibody is directed.

[0070] The present invention also provides a pharmaceutical composition comprising a recombinant polypeptide. For example a recombinant polypeptide of the present invention may be used for drug-targeting. The homologous sequence which is incorporated into the hybrid  $\beta$ -lactamase protein may be specifically selected from those determinants which are bound by cellular receptors (for example of cancerous cells or cells infected by a virus). Preferably a (therapeutically) inactive pro-drug is used, which is activated through cleavage by the  $\beta$ -lactamase moiety into an active drug. Then such cellular targets which are involved in a disease can be inhibited or destroyed. The present invention also provides the use of a recombinant polypeptide for the manufacture of a medicament for the preventive and/or therapeutic treatment of diseases selected from the group cancer, viral diseases and bacterial diseases (or infection diseases), autoimmune diseases and allergy.

[0071] The present invention also provides a method for screening a compound for treatment, prevention and/or diagnosis of a disease which comprises the step of detecting the interaction between the homologous sequence of the hybrid  $\beta$ -lactamase according to the present invention and a protein or polypeptide which binds to the homologous sequence in the presence of a compound to be tested. Preferably the compound to be tested is selected as the candidate of an effective

medicament wherein the compound has an effect on the interaction between the homologous sequence inserted into the hybrid  $\beta$ -lactamase and the peptide which binds to the homologous sequence.

[0072] In a particularly preferred embodiment the method comprises the steps of:

- a) subjecting the recombinant polypeptide of the present invention and a polypeptide which binds to the homologous sequence to interaction with each other in the presence of the compound to be tested;
- b) subjecting the recombinant polypeptide of the present invention and a polypeptide which binds to the homologous sequence to interaction with each other in the absence of the compound to be tested;
- c) detecting the interactions in the steps a) and b), and
- d) comparing the interactions in the steps a) and b) to choose the compound having an effect on the interaction as a candidate of an effective medicament.

[0073] The present invention further provides a biological sensor comprising a recombinant polypeptide of the present invention. The term biosensor has been applied to devices either (1) used to monitor living systems, or (2) incorporate biologic or biomimetic elements. The consensus, however, is that the term should be reserved for use in the context of a sensor incorporating a biological element such as an enzyme, antibody, nucleic acid, microorganism or cell. The term "biosensor" as used in this patent application will be defined as:

analytical devices incorporating a biological material or a biomimetic material (e.g. tissue, microorganisms, organelles, cell receptors, enzymes, antibodies, nucleic acids etc.), intimately associated with or integrated within a physicochemical transducer or transducing (micro)system, which may be optical, electrochemical, thermometric, piezoelectric or magnetic.

[0074] The usual aim of a biosensor is to produce either discrete or continuous digital electronic signals which are proportional to a single analyte or a related group of analytes.

[0075] In a preferred embodiment antibodies are immobilised on a conductive polymeric material. The hybrid protein carrying as homologous sequence an epitope which is specifically recognized and bound by the antibody is used for detecting the respective antibody. Upon cleavage of the substrate by the  $\beta$ -lactamase moiety protons will be generated which can be detected by potentiometric measurement. In an alternative embodiment the antigen is immobilised on the conductive polymeric material. By the use of a hybrid  $\beta$ -lactamase class A protein, wherein the heterologous sequence is binding to the Fc region of antibodies, the presence of antibodies directed to the immobilised antigen can be measured upon cleavage of the substrate and the generation of protons which again are detected by potentiometric measurement.

[0076] In a bid to validate the various fields of application, several hybrid proteins were constructed by inserting restriction sites in the DNA sequences of the TEM-1  $\beta$ -lactamases, BlaP and BlaL. Belonging to the class A group of  $\beta$ -lactamases, these enzymes originate from *Salmonella thyphimurium*, *Bacillus licheniformis* and *Streptomyces cacaoi* respectively. The restriction sites were inserted in a region common to class A  $\beta$ -lactamases, so as to be able to retain  $\beta$ -lactamase activity after large exogenous sequences have been internalised.

[0077] Various exogenous nucleotide sequences were internalised in these recombinant genes. The hybrid genes produced during these operations provide a means of producing various bifunctional proteins, for example:

1. A hybrid protein of the TEM-1  $\beta$ -lactamase where the STa heat stable enterotoxin protein of *Escherichia coli* is internalised (TEMSTA).
2. Hybrid proteins of the TEM-1  $\beta$ -lactamase where 1 to 3 repeated domains of the *Staphylococcus aureus* protein A are internalised (TEM-PA).
3. Hybrid proteins of the BlaP  $\beta$ -lactamase where 1 to 3 repeated domains of the *Staphylococcus aureus* protein A are internalised (BlaP-PA).
4. Hybrid proteins of the TEM-1  $\beta$ -lactamase where the domain/domains B1 and/or B2 of the protein G of *Streptococcus pyogenes* are internalised (TEM-PG).
5. Hybrid proteins of the BlaP  $\beta$ -lactamase where the domain/domains B1 and/or B2 of the protein G of *Streptococcus pyogenes* are internalised (BlaP-PG).

6. A hybrid protein of the BlaP  $\beta$ -lactamase where a linear antigenic determinant of the hemagglutinin of the Influenza virus is internalised (BlaP-HA).
7. Hybrid proteins of the TEM-1  $\beta$ -lactamase where fragments of human phospholipase - type II, hPLA<sub>2</sub> are internalised (TEM-PLA2).
8. A hybrid protein of the BlaP  $\beta$ -lactamase where fragments of multimerised polypeptides comprising three amino acids repeated in tandem and presenting an affinity for bacterial endotoxins are internalised (BlaP-LPS).
9. A hybrid protein of the  $\beta$ -lactamase AmpC in which one Fc-binding domain of the *Staphylococcus aureus* protein A is internalised (AmpC-PA).
10. A hybrid protein of the  $\beta$ -lactamase BlaR-CTD in which a linear antigenic determinant of the Influenza virus hemagglutinin is internalised (BlaR-CTD-HA).
11. A hybrid proteins of the  $\beta$ -lactamase BlaR-CTD in which one Fc-binding domain of the *Staphylococcus aureus* protein A is internalised (CTD-PA).
12. Use of the hybrid protein BlaP-PA in an electrobiochemical biosensor system.

**[0078] Description of the figures**

Figure 1 shows the 3D structure of TEM-1  $\beta$ -lactamase. A: active site of the enzyme. B: highly tolerant position to exogenous polypeptide insertion.

Figure 2 shows the model of  $\beta$ -lactamase hydrolysis of penicillin substrate.

Figure 3 shows the sequence of restriction cassettes internalised in TEM-1 coding sequence.

Figure 4 shows the 3D structures of TEM-1 and BlaP  $\beta$ -lactamases. Arrows show the polypeptide insertion site.

Figure 5 shows the sequence of *Sma*I and *Eco*RV restriction site introduced in BlaP and BlaL coding sequence, respectively.

Figure 6 shows the fold of the TEM-1  $\beta$ -lactamase. The position of the permissive sites (filled square), the semi-permissive site (open square) and the non-permissive sites (grey square) are indicated.

Figure 7 shows the toxicity titration curve of the hybrid proteins.

Figure 8 shows the immunogenicity determined by ELISA for the TEM197H and TEM197STa. A) The presence of anti-TEM antibodies was estimated by coating 250 ng of TEM per well. B) The presence of anti-STa antibodies was estimated by coating 250 ng of GST-STa per well. The serum was diluted 100 fold in PBS buffer. The numbers below the columns of the diagram indicate different mouse individuals.

Figure 9 shows the titration curve of the anti-TEM IgG in the serum collected at day 56.

Figure 10 shows the isotypic response against the carrier protein (TEM197H). The numbers below the columns of the diagram indicate different mouse individuals.

Figure 11 shows the determination of the level of the anti-TEM IgG raised against TEM197H (1), TEM197STa (2), TEM216STa (3), TEM232STa (4) and TEM260STa (5). The numbers below the columns of the diagram indicate different mouse individuals.

Figure 12 shows the construction of hybrid proteins of the TEM-1  $\beta$ -lactamase wherein one or more repeated domains of the *Staphylococcus aureus* protein A (figure 13 A) are internalised. A: protein A of *Staphylococcus aureus* is composed of five repeated domains indicated by letters E, D, A, B and C. These domains bind the antibody Fc region. S is the signal sequence. The sequence at the C-terminus is the peptidoglycan fixation domain (P). B: shows the structure of the E domain. Each of the repeated domains of protein A is organised into three  $\alpha$  helices. C: the DNA coding for the repeated domains of protein A was amplified by PCR. D: the agarose gel is showing restriction analysis of different hybrid  $\beta$ -lactamase clones bearing 1, 2 or 3 domains of protein A. E: the SDS-PAGE gel analysis shows the hybrid  $\beta$ -lactamase proteins wherein one or three domains of protein A have been incorporated.

Figure 13 shows the titration curve of immobilised rabbit IgG by TEM-PA hybrid protein. The adsorbance is plotted against the amount of fixed rabbit IgG (ng).



Figure 14 shows the construction of the hybrid proteins of the TEM-1  $\beta$ -lactamase where the B1 and/or B2 domain or domains of the *Streptococcus pyogenes* protein G were internalised. A shows that protein G is composed of 2 repeated domains, called B1 and B2 that bind to the antibody Fc region. They confer an affinity for the antibodies Fc region. S is the signal peptide sequence of protein G. B shows that each of the 2 domains is organised with a  $\beta$ -sheet and  $\alpha$ -helices. C shows that the nucleotide sequence encoding for the repeated domains of the G protein were cloned into the TEM-1  $\beta$ -lactamase sequence. D shows an SDS-PAGE of hybrid  $\beta$ -lactamase TEM-1 having 2 domains of protein G internalised.

Figure 15 shows the nucleotide sequence of insertion site of BlaP  $\beta$ -lactamase and BlaP-HA hybrid protein.

Figure 16 shows a 12% SDS-PAGE gel electrophoresis of the BlaP and BlaP-HA  $\beta$ -lactamases after SFF partial purification of periplasmic fractions coming from *E. coli* strain transformed with pROGENO-1 BlaP(211/SmaI) and pROGENO-1 BlaP-HA. Transformed bacteria were grown over night on rich medium at 37°C.

Figure 17 shows a Western Blot analysis of the BlaP and BlaP-HA  $\beta$ -lactamases using monoclonal anti-HA antibody conjugated with peroxydase. Immunorecognised proteins were visualised by enhanced chemiluminescence detection.

Figure 18 shows the titration curve of immobilised rat IgG by BlaP-HA hybrid protein. The absorbance is plotted against the quantity of IgG1 of rat anti-HA in ng.

Figure 19 shows the agarose gel where PCR amplification products of TEM-1 (197/SmaI) and some hybrid TEM-1 hPLA<sub>2</sub> protein were loaded.

Figure 20 shows the primary structure of the hPLA<sub>2</sub> on which the various fragments internalised in TEM-1 are underlined (1,2 and 3).

Figure 21 shows the Potentiometric measurement of a platinum electrode where rabbit antibodies were immobilised on functionalised aniline by succinimidyl group. Curve A: base line Pt/Pani/Pani-R/IgG/TemPA without substrate of the  $\beta$ -lactamase.

Curve B: The release of protons starts with the addition of the substrate (benzylpénicilline) and the electrode potential increases proportionally with the quantity of substrate. Point 1,  $2,6 \cdot 10^{-4}$  M; point 2,  $2,6 \cdot 10^{-3}$  M; point 3,  $2,6 \cdot 10^{-2}$  M; point 4,  $2,6 \cdot 10^{-1}$  M; point 5,  $5,2 \cdot 10^{-1}$  M.

Figure 22 shows the detection threshold between 5 and 100 ng of rabbit IgG binding to the Fc binding domain of Staphylococcus aureus protein A internalised into AmpC  $\beta$ -lactamase according to example 18.

Figure 23 shows binding of fluorescent  $\beta$ -lactam and antibody Fc-domain according to example 20. The BlaR-CTD\_F514-PA of example 20 was acylated or not by fluorescent ampicillin and subsequently immobilized on a membrane. After saturation with non-fat dried milk 3%, Donkey anti-rabbit IgG coupled to horseradish peroxidase (Amersham Bioscience) were added. After washing and addition of ECL Immunodetection reagent (Amersham Bioscience) the slot-blot was revealed after 5 minutes. (A) represents non acylated and (B) represents acylated with Fluorescent ampicillin (B) BlaR-CTD\_F514-PA

lane 1: 0, 0.3, 0.6, 0.9  $\mu$ g of total proteins

lane 2: 1.2, 1.5, 1.8, 2.1  $\mu$ g of total proteins

lane 3: 2.4, 2.7, 3, 5  $\mu$ g of total proteins.

[0079] The present invention will be explained in more detail in the following examples, which however do not limit the scope of protection in any way.

## EXAMPLES

### Methodology for constructing hybrid proteins

**[0080] Example 1: Inserting unique restriction sites in the genes of the TEM-1, BlaP and BlaL  $\beta$ -lactamases so as to internalise exogenous nucleotide sequences.**

**[0081]** The coding sequence of the  $\beta$ -lactamase TEM-1 which was used for internalising exogenous sequences of peptide initially contains a *KpnI* restriction site. In order to broaden the range of DNA fragments to be internalised in the carrier protein, two types of restriction cassettes were inserted into the *KpnI* site (figure 3). Among the new restriction sites, the *SmaI* site produces blunt ends compatible with all nucleotide fragments also having blunt ends. This change provides a means of making out the internalisation of the random nucleotide fragments originating with a gene of interest or the genomic DNA of any organism. *E. coli* production assays have shown that the new TEM-1 hybrid proteins retain their  $\beta$ -lactamase activity after the restriction cassettes have been internalised.

**[0082]** As figure 4 shows, the 3D structure of the BlaP  $\beta$ -lactamase of *B. licheniformis* is very close to that of TEM-1. This specific feature is shared by all  $\beta$ -lactamases of the same class. Owing to their insensitivity to the proteases action, a *SmaI* restriction site was inserted into the BlaP and BlaL coding sequences to allow exogenous nucleotide sequences to be internalised (figure 5). The restriction site was inserted in a region that was the same as the one used to internalise exogenous peptides in TEM-1 (figure 5). By the end of this process, it was possible to show that BlaP and BlaL  $\beta$ -lactamases retained their enzymatic activity.

**[0083] Example 2: Synthesis of hybrid proteins TEM-1 and the thermostable enterotoxin STa of Escherichia coli (TEM-STa)**

**[0084]** STa is a thermostable toxin mainly produced by enterotoxic Escherichia coli (*E. coli*) strains found either in animal as bovine or in human. STa induces severe and lethal diarrhoea in human and new borne calf respectively (Mainil J,2000). The

thermostable enterotoxin STa is a polypeptide of 18 amino acid residues. It exhibits a discrete three-dimensional structure, which is stabilised by the presence of three disulfide bonds (Gariépy et al, 1986; Shimoniski et al, 1987). The native fold of the polypeptide only mediates the toxicity.

[0085] The small size of STa does not allow its recognition by the host immune system and no anti-STa antibodies can be produced. Therefore, no protection of the host and no vertical protection (mother-foetus) are possible.

[0086] In order to develop an immune response against STa, the 18 residues can be linked to a large protein (called the carrier protein). The aim is to design a set of hybrid protein in which the STa peptide will be inserted into the TEM-1 class A  $\beta$ -lactamase scaffold. The different hybrid proteins will be produced in *E. coli* and purified to homogeneity. The different proteins will be injected in mice and the immune response toward the carrier protein and the insert will be studied.

[0087] **Results**

[0088] **Example 3: Selection of permissive sites.**

[0089] When performing the present invention the STa amino acid sequence was introduced into eight different positions of TEM-1. Namely, STa was placed after amino acid residues 37, 197, 198, 206, 216, 218, 232 and 260 of TEM-1 respectively (figure 6).

[0090] Those hybrid proteins were obtained by introducing the DNA coding sequence for the 18 amino acid peptide STa into the gene ampR of pBr322. The different hybrid proteins were produced in *E. coli*. The production of a stable and active enzyme was tested as follow:

[0091] a) Western blot with anti-TEM antibodies were performed to verify the production of the hybrid.

[0092] b) Determination of the MIC (minimal inhibitory concentration) of ampicillin for the different E. coli strains.

[0093] These data indicated that three different types of insertion sites could be defined. The sites in position 197, 198, 216 and 218 are permissive (detection of TEM by western blot and high MIC values). The sites in position 37, 206 and 260 are semi permissive (detection of TEM by western blot and low MIC values). The site in position 232 is a non-permissive site (no TEM-1 production and low MIC). Two positions were selected: The first one is the position 197, which is located on solvent exposed loop, and position 216 located on a buried loop.

[0094] **Example 4: Production of the different hybrid proteins.**

[0095] The hybrid proteins in which the STa sequence was inserted in position 197 and 216 were produced in E. coli. Their corresponding genes were inserted in a pTAC11 vector. The hybrids TEM197STa and TEM216STa were produced at 18°C in LG media. The enzymes were purified in three purification steps (one QFF sephaorse pH 7.5, a QFF sepharose pH 6.5 and a superdex 75 molecular sieve). The purification yield was estimated at 2 mg/liter of culture for the two enzymes.

[0096] The TEM197H (TEM-1 + amino acids inserted in position 197) was also produced in E. coli. The production was performed in a SB media. The culture was incubated at 18°C for 28 h. The enzyme was purified as described above. The purification yield was 12.6 mg/liter of culture.

[0097] Finally, as protein control, the STa sequence was introduced at the C-terminal of the glutathion-S-transferase (GST). The fusion protein was purified by affinity chromatography. The purification yield was 30 mg/liter of culture.

[0098] **Example 5: Biological activity of TEM197 STa and TEM216STa.**

[0099] **1) Beta-lactamase activity.** Table 1 shows the steady state kinetic parameters for the different hybrid proteins and the wild type enzyme. The data indicated that the catalytic efficiencies of the different hybrids are lower than the WT.

Nevertheless, it could be demonstrated that the insertion of the STa moiety does not drastically impaired the catalytic efficiency of the TEM-1 enzyme. The conclusion is that the fold of the TEM-1 is not strongly affected by the presence of STa.

[00100]      **2) Toxicity of the hybrid proteins.** The toxicity of the hybrid proteins were tested by suckling mouse assay (Gianella et al, 1976). The toxicity of STa is due to the secretion of physiological fluid in the bladder. The mass of fluid can be estimated by the determination of the ratio between the weight of the bladder and the weight of the mouse carcasse (I/C). If  $I/C < 0.075$ , no toxic effect is detected. If  $0.075 < I/C < 0.083$  represent an intermediary effect of the toxin while an  $I/C > 0.083$  indicated an strong toxic effect. Three control reactions were made by using a purified STa peptide, a supernatant of E. coli which produce (B44) or not the enterotoxin STa. The data presented in table 2 indicated that the TEM197STa and TEM216STa yielded a toxic activity.

[00101]      **3) Titration of the STa toxicity.** The suckling mouse assay was performed for different protein concentrations of 197STa, 216STa and GST-STa. The I/C values were determined in function of the hybrid protein concentration (figure 7). These data indicated that the toxicity of the TEM197STa and TEM216STa were 200 fold lower than the native STa. Interestingly, the toxicity of the GST-STa was 2000 fold lower compared to STa.

[00102]      **Example 6: Immunization assays.**

[00103]      Six groups of three-month-old BALB/c (H-2d) female mice (Dr. Collard, Department of Animal immunology, Centre d'Economie Rurale) were used for immunisation with the different purified recombinant proteins. The mice were immunised with 50 µg of protein diluted in 50 mM sodium phosphate pH 7.2, 0.1 M NaCl (PBS buffer) containing the QuilA adjuvant (Spikoside, Isotech AB, Luleå, Sweden). Three, six weeks and 16 weeks later (day 21,42 and 112 respectively), the mice were boosted with 50 µg of the same recombinant protein. At two weeks time intervals after the first injection (day 14) and after each boosts (day 35, 56 and 127), sera were collected, pooled per group of mice, and then tested for the presence of anti-TEM and anti-STa antibodies by ELISA (figure 8 A and B). The presence of IgG

anti-TEM was found in the serum collected at day 14 for TEM197STA and TEM216STa and at day 35 in the case of TEM197 H respectively. In addition, the IgG anti-Sta were produced against TEM197STa, TEM216STa and GST-STa. The immune response was detected in all the case after the second boost. The production of antibodies was always higher when the GST-STa was injected into the mice.

**[00104] Example 7: Titration curve of anti-Tem IgG antibodies and isotyping of the immune response.**

**[00105]** Figure 9 shows that, after the second boost, the level of anti-TEM antibodies in the different serum (with the exception of GST-STa) was equivalent. The titre was estimated to be 10000.

**[00106]** The nature of the different antibodies produced against TEM-1 was characterized (figure 10). The nature of the antibodies (IgG1, IgG2, IgG2a, IgG2b, IgA and IgM) was determined by ELISA. The TEM-H was used for this experiment and the day 127 - serum of mice immunised with TEM-H was chosen. The data indicated clearly that the immune response yielded t a strong production of IgG1 and IgG2 antibodies. The IgA and IgM antibodies were poorly expressed. In addition the IgG2 response was further characterised. Furthermore, also the IgG2a and IgG2b were found. These data indicated that the presence of TEM-1 can induced both the Th1 and Th2 immune response.

**[00107] Example 8: Stability of the immune response versus time.**

**[00108]** The level of total anti-TEM IgG was measured by ELISA in serums collected at day 127 and 356 after the first injection. No boosts were realised after day 127. The results (figure 11) showed that the IgG level was always slightly higher in the serum day356 compared to those collected at day 127. Consequently, the immune response is considered to be stable for at least one year after a contact between the mice and the TEM  $\beta$ -lactamase.

**[00109] Example 9: Neutralization of the native STa toxicity.**

**[00110]** The native STa enterotoxin was incubated in presence of diluted (4 to 32 fold) serum containing the antibodies raised against GST-STa, TEM197STa and TEM260STa. The solutions were incubated for 16 hours at 4°C. The residual toxicity of the sample was estimated by "suckling mouse assay" (table 3).

**[00111]** The data indicated that the incubation of STa with the TEM197STa or GST-STa serum allowed a neutralisation of the biological activity of STa. Unfortunately, no clear data could be obtained for the TEM232STa.



**Table 1.** Kinetic parameters of the TEM197STa, TEM216STa, TEM197H and TEM-1

| <b>Antibiotics</b>      | <b>proteins</b> | <b><math>k_{cat}</math> (<math>s^{-1}</math>)</b> | <b><math>K_M</math> (<math>\mu M</math>)</b> | <b><math>k_{cat}/K_M</math> (<math>\mu M^{-1}s^{-1}</math>)</b> |
|-------------------------|-----------------|---------------------------------------------------|----------------------------------------------|-----------------------------------------------------------------|
| <b>Benzylpenicillin</b> | <b>TEM-1</b>    | 1500                                              | 18                                           | 80                                                              |
|                         | <b>197H</b>     | 600                                               | 65                                           | 9                                                               |
|                         | <b>197STa</b>   | 56                                                | DELAY                                        | DELAY                                                           |
|                         | <b>216STa</b>   | 70                                                | DELAY                                        | DELAY                                                           |
| <b>Nitrocefin</b>       | <b>WT</b>       | 930                                               | 52                                           | 18                                                              |
|                         | <b>197H</b>     | 770                                               | 170                                          | 4.5                                                             |
|                         | <b>197STa</b>   | >560                                              | >280                                         | 2                                                               |
|                         | <b>216STa</b>   | N.D.                                              | N.D.                                         | N.D.                                                            |
| <b>Cephaloridine</b>    | <b>WT</b>       | 1500                                              | 670                                          | 2.2                                                             |
|                         | <b>197H</b>     | >1000                                             | >1000                                        | 1                                                               |
|                         | <b>197STa</b>   | 256                                               | 172                                          | 1.5                                                             |
|                         | <b>216STa</b>   | 4.5                                               | 720                                          | 0.0062                                                          |

**Table 2. Determination of the toxicity of the different hybrid proteins.**Positive control: *E. coli* B44 producing STaNegative control: *E. coli* non-producer of STa

| Proteins           | (I/C)              | Toxicity |
|--------------------|--------------------|----------|
| 197H               | $0.0545 \pm 0.001$ | -        |
| 197STa             | $0.107 \pm 0.009$  | +        |
| 216STa             | $0.106 \pm 0.011$  | +        |
| GST-STa            | $0.074 \pm 0.006$  | Int      |
| <i>E. coli</i> B44 | $0.0925 \pm 0.001$ | +        |
| <i>E. coli</i>     | $0.0525 \pm 0.001$ | -        |

**Table 3. Neutralisation of the STa toxicity by the mice serum containing anti-GST-STa, TEM197STa, TEM260STa immuno-globulins.** The toxicity of the serum TEM197STa, TEM197H and the serum collected at day 1 was tested by suckling mouse assay. The I/c ratio was measured with the serum diluted 8 fold. The toxicity of the protein samples TEM197STa and STa was also determined. The toxicity was measured by the I/C ratio. The STa peptide is diluted 16 fold in PBS buffer. The different serum were diluted 4 fold (4x), 8 fold (8x), 16 fold (16x) and 32 fold (32x) respectively. A positive and a negative sign indicate that the solution yielded or not a toxic response respectively.

|                 | Samples           | STa     | I/C             | Toxicity |
|-----------------|-------------------|---------|-----------------|----------|
| <b>Controls</b> | 197Sta            | /       | 0.102           | +        |
|                 | /                 | STa     | 0.093           | +        |
|                 | Serum 197STa 8x   | /       | 0.059           | -        |
|                 | Serum 197H 8x     | STa     | 0.073           | -        |
|                 | Serum day1        | STa     | 0.051           | -        |
| <b>Serums</b>   | <b>Serums</b>     |         |                 |          |
|                 | 4x <b>GST-STa</b> | STa     | 0.060           | -        |
|                 | 8x                | STa     | 0.075           | -        |
|                 | 16x               | STa     | 0.091           | +        |
|                 | 32x               | STa     | 0.084           | +        |
|                 | 4x <b>197STa</b>  | STa     | 0.063           | -        |
|                 | 8x                | STa     | 0.082           | +/-      |
|                 | 16x               | STa     | 0.088           | +        |
|                 | 32x               | STa     | 0.094           | +        |
|                 | 4x <b>216STa</b>  | STa     | 0.120-<br>0.054 | + -      |
|                 | 16x               | STa     | 0.144-<br>0.074 | + -      |
|                 | 32x               | STa 16x | 0.101-<br>0.120 | + +      |

[00112] **Conclusions:** The results indicates that:

- 1) The TEM-1  $\beta$ -lactamase allowed the insertion of large peptide sequences between the helices  $\alpha 8$  and  $\alpha 9$  (position 197) and also between the helices  $\alpha 9$  and  $\alpha 10$  (site 216) without any major loss of activity and stability.
- 2) Different hybrid proteins (TEM197STa, TEM198STa, TEM216STa, TEM260STa, GST-STa and TEM197H respectively) were produced and purified to homogeneity.
- 3) The hybrid proteins TEM197STa and TEM216STa possess a rather high residual catalytic efficiency ( $k_{cat}/K_M > 2 \mu M^{-1} s^{-1}$  for nitrocefin).
- 4) The different hybrid proteins exhibited a reduced toxicity compared to the native STa polypeptide.
- 5) Both Th1 and Th2 response toward the TEM enzyme but also against STa could be detected.
- 6) The serum raised against TEM197STa contains antibodies that allow a neutralisation of the biological activity of the native STa enterotoxin.

[00113] **Example 10: Construction of hybrid proteins of the TEM-1  $\beta$ -lactamase where 1 to 3 repeated domains of the Staphylococcus aureus protein A are internalised (TEM-PA)**

[00114] The aim was to internalise one or more repeated domains of protein A in position 197 of the TEM-1  $\beta$ -lactamase (197/SmaI). Protein A is composed of 5 repeated domains (fig. 12A). Each of the repeated domains of protein A is organised into three  $\alpha$  helices (fig. 12B) interacting with the CH2 and CH3 domains of the Fc part of the IgG. This link is primarily stabilised via hydrophobic interactions. Figure 12 A, B, C.

[00115] In order to amplify the repeated domains of protein A, two primers (5'-TCAGTTAACAATTTCAACAAAGAACAACAAAATGCT-3', SEQ ID NO: 7; 5'-TCGAAATTTTTTGTGTCTTCCTCTTTTGG-3', SEQ ID NO: 8) were created which hybridise at the start and end of the gene encoding the proteins A's domain B. The high similarities between the nucleotide sequences of the 5 repeated domains of

protein A allow the 5 domains to be amplified on the basis of the same set of primers. In the case of the more or less long polymerisation times, several repeated domains may be amplified via the same PCR fragment (fig. 12C). The latter feature allows a bank of nucleotidic sequences to be obtained where the five repeated domains of protein A are coded separately or in association with each others. In order to provide a highest degree of freedom for the correct folding of the domain internalised in the  $\beta$ -lactamase and in a way that reduces the effect of its steric constraints on the carrier protein (TEM-1), two amino acid residues were added on either side of the fragment. Ser Val for the N-terminal part and Phe Arg for the C-terminal end. It is important to stress that these primers are designed so as to amplify a fragment that preserved the reading frame of TEM-1 and of the repeated domains of protein A during the internalisation reaction.

[00116] The structural gene of the protein A, originates from a *Staphylococcus aureus* strain isolated at the Centre for Protein Engineering, was amplified by PCR with the following primers: 5'-CATATGAAAAAGAAAAACATTTATTCAATTCGT-3' SEQ ID NO: 9; 5'- GGATCCTTATAGTTCGCGACGACGTCCAGCTAA-3' SEQ ID NO: 10; and in the following conditions: 95°C-180 sec, 95°C-30 sec, 55°C-60 sec, 72°C-120 sec 30 cycles, mixture of Taq polymerase/Pfu polymerase and cloned into the pGEM-T-easy plasmid.

[00117] The repeated domains of protein A (180-pb) were amplified by PCR (95°C-180 sec, 95°C-30 sec, 60°C-60 sec, 72°C-60 sec 35 cycles; mixture of Taq polymerase/Pfu polymerase) on the entire gene. An analysis of the amplification product for the repeated domains of protein A shows a ladder profile where the size of the amplified fragments is a multiple of 180-pb (fig. 12C). The PCR product was purified from an agarose gel, bunt ended by the action of the Pfu polymerase then dephosphorylated by Calf intestine phosphatase. The library of protein A PCR fragments was shotgun cloned in the TEM-1  $\beta$ -lactamase gene that was cloned beforehand in the expression construct pROGENO-1 and digested by *Sma*I. The pROGENO-1 plasmid allows for a high constitutive expression of the recombinant  $\beta$ -lactamases in *E. coli*. In this plasmid, a unique restriction site recognised by the *Sma*I enzyme is present in position 197 of the TEM-1  $\beta$ -lactamase. After transformation, the bacteria were selected via LB agar plate + Spectinomycin (100

µg/ml final) and cephaloridin (50 µg/ml). The cephaloridin is an antibiotic with a  $\beta$ -lactam ring hydrolysed by the TEM-1 wild-type and recombinant protein.

[00118] After plate selection, a colony PCR reaction analysis was performed to control the size of the TEM-1 gene. Towards this end, primers outcrossing upstream and downstream from the coding sequence of the mature form of TEM-1 (5'-cgggagctcaggctcaccagaaacgctggtg-3'; 5'-cgggaattctcaccaatgcttaatcagtgaggcacc (SEQ ID NO 11 and SEQ ID NO:12); 95°C-180 sec; 95°C-30 sec, 65°C-60 sec, 72°C-90 sec 35 cycles, mixture of Taq polymerase/pfu polymerase) were used. In a population of 30 clones, all of them were bigger than the encoding TEM-1 gene. On the agarose gel shown in figure 12D, the PCR fragments coding for the TEM-PA hybrid proteins have been loaded, where 1 to 3 repeated domains of the protein A were internalised.

[00119] The productions of the various TEM-PA hybrid proteins were achieved in the *E. coli* JM109 strain. After a 24-hours fermentation at 37°C in a rich medium, TEM-PA hybrid proteins were overproduced in the periplasm of the bacteria. However the SDS-PAGE gel analysis shows that the hybrid proteins are partly proteolysed during their biosynthesis. The hybrid proteins were then affinity chromatography purified on IgG-sepharose (fig. 12E) until homogeneity was reached. This showed that the domains of the protein A internalised in TEM-1 retained their affinity for the antibodies Fc region. Hydrolysis tests on the chromogenic substrate nitrocefin (red cephalosporin, antibiotic with a  $\beta$ -lactam ring) reveal that the hybrid proteins also retain  $\beta$ -lactamase activity after purification.

[00120] In order to check if the TEM-PA chimeras can be used to quantify the antibodies, ELISAs were developed in which increasing levels of rabbit IgG were immobilised on a polystyrene microplate by alkaline pH absorption ( $\text{Na}_2\text{CO}_3$  1.59 g/l,  $\text{NaHCO}_3$  2.93 g/l, pH 9.6). After saturation ( $\text{PO}_4^-$  50 mM, NaCl 150 mM, Tween-20 0.05%, Non-fat dried Milk 3%, pH 7.5), a fixed amount of TEM-PA hybrid protein was added ( $\text{PO}_4^-$  50 mM, NaCl 150 mM, Tween-20 0.05%, Non-fat dried Milk 1%, pH 7.5) where one repeated domain of the protein A was internalised. After washing (3X -  $\text{PO}_4^-$  50 mM, NaCl 150 mM, Tween-20 0.05%, Non-fat dried Milk 1%, pH 7.5; 1x -  $\text{PO}_4^-$  50 mM, NaCl 150 mM, pH 7.5), red cephalosporin (100 µM) was added. In this test, the  $\beta$ -lactamase activity gave rise to a red colour which was followed at 482 nm.

Figure 13 shows the possibility of detecting between 10 and 100 ng of rabbit IgG after a 1h development. The sensitivity of this test should be further increased by using a chimera protein containing several repeated domains of the protein A.

**[00121] Example 11 Construction of the hybrid proteins of the BlaP  $\beta$ -lactamase where 1 to 3 repeated domains of Staphylococcus aureus protein A are internalised (BlaP-PA).**

**[00122]** The BlaP-PA chimerical partner were constructed and purified according to the same procedure described in example 10 for the TEM-1  $\beta$ -lactamase. The BlaP  $\beta$ -lactamase is used as carrier of peptide fragment and the exogenous peptides are internalised at the site 211 (211/SmaI). The resulting hybrid  $\beta$ -lactamase retains its activity and also the internalised protein A is functional.

**[00123] Example 12 Construction of the hybrid proteins of the TEM-1  $\beta$ -lactamase where the B1 and/or B2 domain or domains of the Streptococcus pyogenes protein G are internalised (TEM-PG).**

**[00124]** The aim was to internalise one or more repeated domains of the protein G in position 197 of the TEM-1  $\beta$ -lactamase (197/SmaI). The protein G is composed of 2 repeated domains, called B1 and B2. They confer an affinity for the antibodies Fc region (fig. 14A). Each of the two domains is organised with a  $\beta$  sheet and a  $\alpha$  helices (fig. 14B) interacting with the CH2 and CH3 domains of the Fc region of the IgG.

**[00125]** The methodology used to construct TEM-PG hybrid proteins is exactly the same as the one described in example 10 for TEM-PA, apart from the following observations:

**[00126]** The primers used are:

5'-GGCTGTA CT TACAAATTAATCCTTAATGGTAAAACATTG-3' (SEQ ID NO: 13) and 5'-CTCTCTTTTCAGTTACCGTAAAGGTCTTAGTCGC-3' (SEQ ID NO: 14). The structural gene used as a matrix during the PCR originates from the genomic DNA of *Streptococcus pyogenes* strain isolated at the Centre for Protein Engineering. In order to reduce the steric constraints the following amino acid residues were added

on either side of the fragment. Gly Cys for the N-terminal part and Arg Glu for the C-terminal end.

[00127] At the end of the screening stages, TEM-1  $\beta$ -lactamases was isolated where 1 or 2 repeated domains of the protein G were internalised (fig. 14C). The affinity of the TEM-PG chimera proteins for the IgG immobilised on the sepharose column shows that the internalised domains of proteins G are always functional (fig. 14E). Hydrolysis tests on the chromogenic substrate nitrocefin (antibiotic with a  $\beta$ -lactam nucleus) show that the TEM-PG chimeras purified on IgG-sepharose retain the  $\beta$ -lactamase activity. The tests showed that the internalised domains of protein G were functional.

[00128] **Example 13 Construction of the hybrid proteins of the BlaP  $\beta$ -lactamase where the B1 and/or B2 domain or domains of the Streptococcus pyogenes protein G are internalised (BlaP-PG).**

[00129] The BlaP-PG chimera proteins were constructed and purified according to the same procedure as the one described in example 11 and 12. The BlaP  $\beta$ -lactamase (211/SmaI) was used as a carrier protein. The chimeras purified on IgG-sepharose retain the  $\beta$ -lactamase activity. The tests also showed that the internalised domains of protein G were functional.

[00130] **Example 14 Construction of the hybrid proteins of the BlaP  $\beta$ -lactamase where a linear epitope of the Influenza virus hemagglutinin is internalised (BlaP-HA)**

[00131] In order to create this hybrid protein, complementary primers (5'-AGGTTTTATCCATACGACGTCCCGGACTACGCCACAACT-3' SEQ ID NO: 15, 5'-AGTTGTGGCGTAGTCCGGGACGTCGTATGGATAAAACCT-3' SEQ ID NO: 16) were created that code for a linear epitope (HA) of the Influenza virus hemagglutinin (YPYDVPDYA). Here and there on the coding region for the epitope, two codons which code for Arg Phe and Thr Thr amino acids were added at the beginning and at the end of epitope, respectively. In this experiment, the polypeptide internalised in BlaP comprises 15 amino acids 4 of which are used for steric constraints (fig. 15).



[00132] The two primers were hybridised and inserted into the BlaP gene (211/SmaI) which was cloned beforehand in the pROGENO-1 expression vector and digested by *SmaI*. After transformation, the bacteria were selected on LB agar plate + Spectinomycin (100 µg/ml final) and cephaloridin (50 µg/ml). At the end of the screening stage, BlaP  $\beta$ -lactamases were isolated where the epitope HA was internalised. The BlaP-HA hybrid protein was then overproduced in *E. coli* as a result of the pROGENO-1 expression vector. After extracting the periplasmic fraction by cold osmotic shock, the BlaP-HA chimera was partly purified on S-sepharose Fast Flow (SFF) in sodium acetate buffer (20 mM, pH 4.5) and eluted with a NaCl gradient. The SDS-PAGE gel featured in figure 16 shows the BlaP and BlaP-HA hybrid protein after SFF purification.

[00133] The antigenicity of the HA epitope internalised in BlaP was controlled by a Western Blot reaction using a specific monoclonal antibody of this hemagglutinin's epitope (rat anti-HA IgG1, 3F10, Roche). Figure 17 shows that the monoclonal anti-HA antibody recognised the denatured BlaP-HA chimera and no cross-reaction with wild-type BlaP was detected.

[00134] The aforementioned Western Blot experiment shows that the HA epitope is recognised when the BlaP-HA chimera protein is denatured. In order to check that the anti-HA antibody also recognises the HA epitope when it is internalised in a non-denatured BlaP form an ELISA reaction was performed in which increasing quantities of rat anti-HA IgG1 were immobilised (not linked to the peroxidase, 3C10, Roche) ( $\text{Na}_2\text{CO}_3$  1.59 g/l,  $\text{NaHCO}_3$  2.93 g/l, pH 9.6). After saturation (Tris 50 mM, NaCl 0.5 M, Tween-20 0.05%, Non-fat dried Milk 1%, pH 7.6), a fixed amount of BlaP-HA chimera protein was added (Tris 50 mM, NaCl 150 mM, Tween-20 0.05%, Non-fat dried Milk 1%, pH 7.6). After washing (3x - Tris 50 mM, NaCl 0.5 M, Tween-20 0.05%, Non-fat dried Milk 1%, pH 7.6; 1x - Tris 50 mM, NaCl 0.5 M, pH 7.6), red cephalosporin (100 µM) was added. Figure 18 shows the possibility of detecting between 10 and 100 ng of rat anti-HA IgG1 after 1h of development. This demonstrates that  $\beta$ -lactamase activity can be used to quantify interaction between antibody and antigen/epitope fragment.

**[00135] Example 15 Construction of the hybrid proteins of the TEM-1  $\beta$ -lactamase where fragments of human phospholipase - type II, hPLA<sub>2</sub> are internalised (TEM-PLA<sub>2</sub>)**

**[00136]** In order to internalise random fragments of the human phospholipase - type II gene, hPLA<sub>2</sub> in the TEM-1  $\beta$ -lactamase, the hPLA<sub>2</sub> gene was amplified by PCR. The PCR reaction was applied with the following primer: 5'-CTCGAGAAAAGAAATTTGGTGAATTTCCAC-3' (SEQ ID NO: 17) and 5'-GCAACGTGGAGTGCTCCCTCTGCAGTGTTT-3' (SEQ ID NO: 18) (95°C-180 sec; 95°C-30 sec, 65°C-60 sec, 72°C-60 sec 35 cycles, mixture of Taq polymerase/Pfu polymerase). After purification, the PCR product was digested with DNase so as to produce DNA fragments between 50 and 430 bp. This stage may be replaced by a nebulisation reaction. The DNA fragments were then purified, blunt ended by the action of the Pfu polymerase then dephosphorylated by Calf intestine phosphatase and shotgun cloned in the TEM-1  $\beta$ -lactamase carried by the expression vector pROGENO-1. At the end of the screening stages, several TEM-1  $\beta$ -lactamases chimeras were isolated where fragments of varying sizes originated from the hPLA<sub>2</sub> gene were internalised (figure 19). In the case of three of them, sequencing reaction was applied in order to identify the hPLA<sub>2</sub> regions which were internalised (figure 20). In the case of the first chimera, the N-terminal domain of the hPLA<sub>2</sub> (residues 1 to 45) had been internalised. This peptide fragment contains the calcium binding site and some residues of the active site. In the second chimera, an internal fragment of the hPLA<sub>2</sub> (residues 40 to 66), covering the residues of the active site were internalised. In the third chimera, all the hPLA<sub>2</sub> protein, except for the 20 last residues, were internalised (figure 20). It is important to stress that the  $\beta$ -lactamase activity is retained for each one of the chimera selected. Trials involving the binding of calcium and measuring phospholipase activity were successful.

**[00137] Example 16 Construction of a hybrid protein of the BlaP  $\beta$ -lactamase where fragments of multimerised polypeptides comprising three amino acids repeated in tandem and presenting an affinity for bacterial endotoxins are internalised (BlaP-LPS)**

**[00138]** In order to construct a new LPS-binding peptide, first of all two complementary primers corresponding to the LPS-binding amino acid sequence (Pro

Ile Ile Lys Leu Leu Lys Leu Leu Lys Leu Leu Arg Arg Lys Leu Leu Lys Leu Leu Lys Leu Leu Pro Asp Gln Glu Phe Lys Gln ) were hybridised. Primer sequence: 5'-CCGATCATCAAACCTTCTCAAGCTGCTTAACTCCTGCGCCGGAACTTCTCAAGCTGCTTAACTCCTGCCGGATCAGGAGTTTAAGCAG-3' and 5'-CTGCTTAACTCCTGATCCGGCAGGAGTTTAAGCAGCTTGAGAAGTTTCCGGCGCAGGAGTTTAAGCAGCTTGAGAAGTTTGATGATCGG-3'. Hybridisation is achieved by heat denaturation followed by a slow cooling stage. Double stranded oligonucleotide was inserted in the gene of the BlaP  $\beta$ -lactamase that was cloned beforehand in the expression vector pROGENO-1 and digested by SmaI. After transformation, the bacteria were selected on LB agar plate + Spectinomycin (100  $\mu$ g/ml final) and cephaloridin (50  $\mu$ g/ml). At the end of the screening stages, BlaP  $\beta$ -lactamases were isolated where LPS-binding domain was internalised. The affinity of the BlaP-LPS chimera proteins for LPS is now being characterised. Hydrolysis tests on the chromogenic substrate nitrocefin reveal that the BlaP-LPS chimeras also retain  $\beta$ -lactamase activity.

**[00139] Example 17 Exploitation of the hybrid protein TEM-PA in electrobiochemical biosensor system**

[00140] The term biosensor has been applied to devices either used to monitor living systems, or to incorporate biologic or biomimetic elements. Here, in this application a "biosensor" is used in the context of a sensor incorporating a biological element such as an enzyme, antibody, nucleic acid, microorganism or cell.

[00141] The usual aim of a biosensor is to produce either discrete or continuous digital electronic signals which are proportional to a single analyte or a related group of analytes.

[00142] Experimental procedure: A polyaniline (Pani) film is electropolymerised on a platinum foil (1 x 0.5 cm) on the basis of a 1 M HClO<sub>4</sub> solution containing 0.1 M aniline, by potential sweeps between -0.2 and 0.8 V/SCE to 20 mV/s. The Pani film is functionalised in an electrochemical bath containing a 1 M HClO<sub>4</sub> solution, 0.05M in 3-aminophenol and 0.05 M aniline with potential sweeps between -0.2 and 0.8 V/SCE to 20 mV/s. The film is then immersed in an acetonitrile solution (4 ml) containing 0.2

ml of triethylamine, 0.04 g of disuccinimidyl carbonate and 0.01g of dimethylaminopyridine (DMAP) for one night at ambient temperature.

[00143] The rabbit antibodies (IgG) are immobilised on the functionalised film for one night in a pH=8 phosphate buffer and 300 µl of a 4mg/ml IgG solution. The IgG assay is achieved as follows: 50 µl of a solution of the TEM-PA hybrid protein (1µg/µl) are deposited on the electrode (Pt/Pani/Pani-R/IgG) for 15 min, the electrode is rinsed by 3 x 5 ml pH=8 phosphate buffer. The potentiometric measurement is achieved in a simple compartment cell containing 4.5 ml 0.1M NaCl solution and a calomel reference electrode (SCE). The working electrode and reference are connected to a multimeter and all the potential values are collected every 30 seconds (fig. 21). Benzylpenicillin is added every minute so that each addition produces a substrate concentration in the bath ranging from  $2.6 \cdot 10^{-4}$  M to  $2.6 \cdot 10^{-1}$  M.

[00144] **Example 18: A hybrid protein of the β-lactamase AmpC in which one Fc-binding domain of the Staphylococcus aureus protein A is internalised (AmpC-PA).**

[00145] In this example a hybrid protein of the β-lactamase AmpC is constructed having one Fc-binding domain of the Staphylococcus aureus protein A internalised (AmpC-PA).

[00146] The blunt end restriction site Scal (AGT ACT) was introduced between the Leu241 and the Asp242 position. E. coli production assays have shown that the new AmpC hybrid protein retains its β-lactamase activity after the restriction site (which represents the codons for Ser and Thr on the protein level) has been internalised.

[00147] The AmpC-PA hybrid partners were constructed and purified according to the procedure described in example 10 for β-lactamase TEM-1 in the present patent. The resulting hybrid β-lactamase retains its enzymatic activity and also the internalised protein A is functional as noted after affinity chromatography on IgG-sepharose. This showed that the internalised domains of the protein A retained their affinity for the Fc region of the antibodies suggesting that they are correctly folded.

The AmpC-PA hybrid protein was used to quantify coated antibodies by ELISA method. Rabbit IgG were coated on a polystyrene microplate by alkaline pH absorption ( $\text{Na}_2\text{CO}_3$  1.59 g/l,  $\text{NaHCO}_3$  2.93 g/l, pH 9.6). After saturation ( $\text{PO}_4^-$  50 mM, NaCl 150 mM, Tween-20 0.05%, Non-fat dried Milk 3%, pH 7.5), a fixed amount of AmpC-PA hybrid protein was added ( $\text{PO}_4^-$  50 mM, NaCl 150 mM, Tween-20 0.05%, Non-fat dried Milk 1%, pH 7.5). The hybrid protein used in this assay contained one repeated domain of the protein A. After washing (3X  $\text{PO}_4^-$  50 mM, NaCl 150 mM, Tween-20 0.05%, Non-fat dried Milk 1%, pH 7.5; 1x  $\text{PO}_4^-$  50 mM, NaCl 150 mM, pH 7.5), 150  $\mu\text{l}$  of nitrocefin (100  $\mu\text{M}$ ) was added. In this test, the  $\beta$ -lactamase activity gave rise to a red colour which was followed at 482 nm. Figure 22 shows a detection threshold between 5 to 100 ng of rabbit IgG after 30 min development.

[00148] **Example 19: A hybrid protein of  $\beta$ -lactamase BlaR-CTD wherein a linear antigenic determinant of the hemagglutinin of the Influenza virus is internalised (CTD-HA).**

[00149] The BlaR protein is the penicillin receptor involved in the induction of the *Bacillus licheniformis* BlaP  $\beta$ -lactamase. The C-terminal domain of BlaR (256 last residues) named BlaR-CTD acts as a penicillin sensor and forms with  $\beta$ -lactam antibiotics a very stable acyl-enzyme compound. BlaR-CTD sequence (256 residues) compared to class D Oxa-2  $\beta$ -lactamase (255 residus) shows 36 % identity. The superposition of the 3D structures showed that these two proteins share the same folding confirming that BlaR-CTD belongs to class D  $\beta$ -lactamase family. The BlaR-CTD advantage is to have a very low deacylation kinetic constant that allows fixing BlaR-CTD on a surface covered with a  $\beta$ -lactam.

[00150] For the construction a 45 pb fragment containing the DNA sequence coding for the HA peptide flanked by two linkers of 3 residues was introduced. In the final construct (BlaR-CTD\_F514-HA), two restriction sites *Bam*HI and *Kpn*l are introduced between the codon coding for Phe514 and the Lys515 in the BlaR-CTD gene. The SEQ ID NO 37 shows the inserted sequence of HA peptide including the linker covering the BamHI and KpnI site, respectively.

[00151] *Bacillus subtilis* production assays have shown that the new BlaR-CTD\_F514-HA hybrid protein retains its capacity to be acylated by  $\beta$ -lactam antibiotic and the HA peptide can be recognized by rabbit anti-HA monoclonal antibodies. The same 45 pb DNA fragment has been inserted in 3 other sites of BlaR-CTD (between E511 and F512, N532 and G533, A561 and D562). Those 3 other sites are also permissive for a HA peptide insertion. These experiments represent the first results showing permissive sites in class D  $\beta$ -lactamase family.

[00152] **Example 20: Hybrid proteins of  $\beta$ -lactamase BlaR-CTD wherein one repeated domain of the *Staphylococcus aureus* protein A was internalised (BlaR-CTD-PA).**

[00153] A 42 pb *Bam*HI-*Kpn*I fragment of the BlaR-CTD\_F514-HA gene was substituted by a 204 pb *Bam*HI-*Kpn*I fragment containing the coding sequence for one repeated domain of the protein A (PA) in order to generate BlaR-CTD\_F514-PA gene. The hybrid protein has been produced by a recombinant *Bacillus subtilis* strain and exported in the extracellular medium. In crude extracellular extract, BlaR-CTD\_F514-PA hybrid retains its capacity to bind fluorescent ampicillin (fluorescent  $\beta$ -lactam) and antibody FC-domain as shown in figure 23.

[00154] The BlaR-CTD\_F514-PA acylated or not by fluorescent ampicillin was immobilized on a membrane by slot-blot experiment. After saturation with non-fat dried milk 3%, Donkey anti-rabbit IgG coupled to horseradish peroxidase (Amersham Bioscience) were added. After washing and addition of ECL Immunodetection reagent (Amersham Bioscience) the slot-blot was revealed after 5 minutes. In the figure, (A) represents non acylated (A) and (B) represents acylated with Fluorescent ampicillin BlaR-CTD F514-PA with fluorescent ampicillin.

[00155] **References**

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